IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY et al.

Serial No.: 09/096,648 Group Art Unit: 1632

Filed: June 12, 1998 Examiner: Martin, J.

For: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

DECLARATION PURSUANT TO 37 C.F.R. §1.132

The Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DM Considered 6/14/00

I, Carl Perez declare as follows:

1) I am currently Director of Projects at Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A IW9. I have held this position since March 10, 1997. I earned a doctoral degree in biophysics at the University of California at Berkeley in August 1984.

2) In my position at Chromos Molecular Systems, Inc., (hereinafter Chromos) I have been extensively involved in projects designed to generate transgenic animals using satellite artificial chromosomes. Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have generated transgenic mice by microinjection of 60 Mb murine satellite DNA-based artificial chromosomes containing multiple copies of the *lacZ* (β-galactosidase) and *hph* (hygromycin phosphotransferase) genes into the pronucleus of mouse zygotes.

Fluorescence *in situ* hybridization (FISH) analysis of preimplantation embryos injected with satellite DNA-based artificial chromosomes demonstrated that 44% of the analyzed embryos contained intact satellite DNA-based artificial chromosomes in 8-67% of the total cells analyzed for each positive embryo. In β-galactosidase staining assays of injected preimplantation embryos at various developmental stages, 31% of the analyzed embryos showed X-gal staining indicating the presence of a functional marker gene in the artificial chromosomes

that was expressed in a mosaic pattern. Seven percent of the mice born after implantation of injected embryos into pseudopregnant female mice were positive for the *hph* gene in nucleic acid amplification analyses of tail DNA. The presence of intact satellite DNA-based artificial chromosomes in mitogen-activated peripheral blood lymphocytes from a female transgenic founder was confirmed by FISH analysis. This analysis also revealed that the artificial chromosomes were maintained as discrete chromosomes in approximately 60% of the cells analyzed and that they had not integrated into the endogenous chromosomes.

Mating of the transgenic female founder with wild-type F1 males yielded progeny, 46% of which were positive for the presence of the *hph* gene in nucleic acid amplification assays of tail DNA. FISH analysis of peripheral blood lymphocytes from progeny carrying the satellite DNA-based artificial chromosomes revealed that intact artificial chromosomes were present in approximately 60% of the analyzed cells with no apparent translocation of the artificial chromosome DNA onto the host chromosomes.

The results of these analyses demonstrate that satellite DNA-based artificial chromosomes as described in the above-referenced application can be used in standard methods of transgenic animal generation to yield viable transgenic animals containing within their cells intact, heterologous gene-containing artificial chromosomes as autonomous, stably replicating, extrachromosomal elements. Furthermore, the results of these analyses demonstrate that the satellite DNA-based artificial chromosomes are transmitted through the germline.

A description of the above-referenced methods, animals and results follows.

I. Materials and methods

A. Satellite DNA-Based Artificial Chromosomes

Satellite DNA-based artificial chromosomes were obtained from a mouse-hamster-human cell line containing a 50-60 Mb micro-megachromosome

carrying the anti-HIV *gag* ribozyme and the hygromycin phosphotransferase and β-galactosidase genes. The cell line was generated in accordance with methods described in detail in the above-referenced application.

Specifically, the H1xHe41 cell line (mouse-hamster-human hybrid cell line carrying a megachromosome and a single human chromosome with CD4 and neo^r genes), which is described in the above-captioned application on page 68, lines 9-15, was subjected to repeated BrdU treatment followed by single cell cloning to yield the mM2C1 cell line. As described in the above-referenced application, the H1xHe41 cells are ultimately derived from EC3/7C5 cells that had been co-transfected with pCH110 and pH132. These plasmids carry the β-galactosidase-encoding gene (*lacZ*), which is linked with the SV40 promoter, (pCH110) and the hygromycin-resistance gene (*hph*) and anti-HIV *gag* ribozyme under control of the β-actin promoter (pH132; see pages 53-54 of the above-captioned application for a description of construction of pH132).

The mM2C1 cell line contains the ~60 Mb megachromosome containing the anti-HIV *gag* ribozyme and the *hph* and *lacZ* genes. Microcells of mM2C1 cells were fused with chinese hamster ovary (CHO) cells by microcell fusion to generated CHO-E4-20 cells as follows. Mitotic cells were harvested from colchicine-treated mM2C1 cells and centrifuged through a percoll gradient in the presence of cytochalasin-B. Microcells were passed through successive filters and overlaid for 20 minutes onto recipient CHO cells and treated with polyethylene glycol. Selection for clones containing satellite DNA-based artificial chromosomes was based on expression of β-galactosidase using hygromycin resistant growth techniques. Single-cell cloning by limiting dilution was repeated several times to yield the CHO-E4-20 cell line that contains two intact, functional ~60 Mb megachromosomes. The cell line was grown under standard conditions in MEM-α medium under selective (0.15 μg/ml hygromycin) conditions.

B. Isolation and Purification of Satellite DNA-Based Artificial Chromosomes

The satellite DNA-based artificial chromosomes were purified from CHO-E4-20 cells using flow cytometry generally as described in the above-referenced application. The artificial chromosomes were first isolated from the cells as follows. Cells were plated in 150 mm tissue culture dishes and supplemented with fetal calf serum and hygromycin B. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0 μg/ml) for 7 hours before harvest. Mitotic cells collected by washing were swollen in a hypotonic buffer of 75 mM KCl for 10 min at room temperature. After swelling, the cells were transferred to the polyamine buffer (80 mM KCl, 70 mM NaCl, 0.1% β-mercaptoethanol, 15 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 0.2 M spermine, 0.5 M spermidine, and 0.25% Triton X-100, adjusted to pH 7.2) and incubated on ice. Shearing of the cell membranes was achieved by gently drawing the cell suspension up and down a 22-gauge needle attached to a 10-ml syringe. Hexylene glycol (2%)/200mM glycine buffer was added to an equal volume of the polyamine buffer containing the released chromosomes giving a final volume of 20 ml. Prior to staining, the chromosome preparation was centrifuged at 100g for 1 minute to remove cellular debris.

The chromosome suspension (supernatant) was removed to a fresh tube and stained with Hoechst 33258 (2.5 μg/ml), chromomycin A3 (50 μg/ml) in the presence of 2.5 mM $MgCl_2$. Samples were stored at 4°C for a minimum of 2 hours. Fifteen minutes before flow cytometric sorting, 10 mM sodium citrate and 25 mM sodium sulfite were added. A final concentration of 15-20 million chromosomes per ml was achieved. All chromosome preparations were filtered through a 35-μm nylon mesh and stored on ice until sorted.

Purification of the satellite DNA-based artificial chromosomes from the chromosome preparation was performed on a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a Turbo-Sort Option and two Innova 306 lasers (Choerent, Palo Alto, CA).

Hoechst 33258 was excited with the primary UV laser beam and excitation detected in FL1 using a 420-nm band-pass filter, whereas chromomycin A3 was excited by the second laser set at 458 nm, and fluorescence detected in FL 4 by using a 475 nm long-pass filter. The sheath buffer used in the sorting procedure contained 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine and 70 μM spermidine. Flow-sorted satellite DNA-based artificial chromosomes were pelleted by centrifugation of a 1-ml sample containing $\sim 10^6$ chromosomes at 2500 x g for 15 minutes at 4°C, and finally retaining only 10-20 μl of loose pellet.

C. Culture of Murine Embryos

Mouse zygotes were collected from superovulated (C57BL/6 x CBA) F1 females approximately 12 hr after fertilization. All mice were viral antibody-free and maintained in a barrier facility according to University of British Columbia guidelines. Harvesting, culture, and implantation of embryos were done using standard procedures [see, e.g., Hogan *et al.* (1994) *Manipulating the Mouse Embryo: A Laboratory Manual.* New York: Cold Spring Laboratory Press].

D. Microinjection of Satellite DNA-Based Artificial Chromosomes

For murine pronuclear injections, non-filamented borosilicate glass micropipettes (Pyrex, Corning No. 77, 1.0 mm O.D. x 0.75 mm I.D.) were pulled and bevelled using a Sutter Model P-97 micropipette puller and a Sutter Model BV-10D micropipette beveler fitted with a 104F fine diamond plate, respectively. Zygotes were deposited in a 12 μl volume of injection medium on a depression microscope slide. The injection medium consisted of one part concentrated satellite DNA-based artificial chromosomes in sheath buffer and 3 parts M2 medium (Sigma), and was covered by mineral oil. Pronuclei and satellite DNA-based artificial chromosomes (approximately 2 μm x 1 μm) were visualized using Leica differential interference contrast optics. One or more artificial chromosomes were frontloaded into the tip of the needle by applying suction on the microinjection needle using a manual air-driven SAS11/2-E equilibrating

syringe (Research Instruments, Cornwall, England), and injected into the male pronucleus.

E. Genomic DNA Extraction, PCR and β-galactosidase Staining

Mouse tail biopsies were obtained under anesthesia at weaning. Total DNA from mouse tails or parts of newborn mice were extracted according to Hogan *et al.* (1994). DNA (400 ng) was amplified using AmpliTaq kit (Perkin Elmer, Foster City, CA). Each 50 μl reaction contained 1x PCR Buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each primer, and 2.5 units of AmpliTaq Gold™ DNA polymerase (Perkin Elmer). The presence of hygromycin phosphotransferase transgene was detected by amplification of an internal 414-bp fragment using the following primers:

5′-CGGGGGCAATGAGATATGAAAAAG-3′ and

5′-GAACCCGCTCGTCTGGCTAAG-3′.

The PCR reaction mixtures were heated to 95°C for 10 min. and subjected to 35 cycles of amplification (94°C 1 min., 59.3°C 1 min., 72°C 1 min.), followed by 10 min. at 72°C. Embryos were assayed for β-galactosidase activity according to Takeda and Toyoda [(1991) *Mol. Reprod. Dev. 30*:90-94].

F. Fluorescence in situ Hybridization

Metaphase spreads from murine embryos were made based on the technique of Garside and Hillman [(1985) *Experientia 41*:1183-1184]. Embryos were arrested in M16 media containing 5 μg/ml colcemid (Sigma) for about 1 hour or 3 μg/ml nocodazole (Sigma) for 12 to 16 hours at 37°C in 5% CO_2. Metaphase spreads from peripheral blood lymphocytes were done according to standard procedures [Dracopoli *et al.* (1994) *Current Protocols in Human Genetics*, New York: John Wiley & Sons]. 100 μl of blood was aseptically collected from each live mouse by saphenous vein bleeding [Hem *et al.* (1998) *Lab. Anim. 32*:364-368], and cultured for three days in a humidified atmosphere at 37°C/5% CO_2 in RPMI 1620 (Gibco/BRL) supplemented with 20% Fetal bovine serum, 3 μg/ml concanavalin A (Type IV-S, Sigma), 10 μg/ml lipopolysaccharide (Sigma), and 5 x 10^{-5}M β-mercaptoethanol (Gibco BRL).

Colcemid was added to a concentration of 0.25 μg/ml and the culture was further incubated at 37°C/5% CO_2 for 2 hours. Mouse major satellite DNA, *lacZ* or *hph* probes were labelled using the Biotin-Nick Translation Mix (Boehringer Mannheim) or the DIG-Nick Translation Mix (Boehringer Mannheim). Fluorescence *in situ* hybridization (FISH) was done as described [Pinkel *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*:2934-2938]. At least 100 lymphocytes were scored at 95% confidence levels as calculated by applying binomial statistics.

II. Analysis of Injected Mouse Embryos

A. Survival

Up to half of all the injected murine embryos survived pronuclear injection of the satellite DNA-based artificial chromosomes (Table 1). Over 50% of the viable artificial chromosome-injected zygotes developed to the blastocyst stage and 47% (14/30) of control buffer-injected embryos reached the blastocyst stage, indicating that the artificial chromosomes did not adversely affect early embryo development.

Table 1. Murine embryo survival and development after pronuclear injection of satellite DNA-based artificial chromosomes

Experiment No.	Viable Embryos/Total Injected	2-Cell Embryos on Day 2 of Culture	Blastocysts on Day 4 of Culture
1	4/31 (31%)	3/4 (75%)	2/4 (50%)
2	8/24 (33%)	4/8 (50%)	4/8 (50%)
3	21/44 (48%)	19/21 (90%)	13/21 (62%)

B. Presence of Satellite DNA-Based Artificial Chromosomes

To look for the presence, and to evaluate the fate of injected satellite DNA-based artificial chromosomes, FISH was performed on chromosome spreads of preimplantation embryos injected with artificial chromosomes. Morulae or blastocysts were probed with *lacZ*. The satellite artificial chromosome was present in 44% of murine embryos analyzed. Embryos exhibited varying degrees of mosaicism for the presence of satellite DNA-based

artificial chromosomes (Table 2), which was detected in 8% to 67% of cells scored for each positive embryo. Translocation events between the artificial chromosome marker sequences and native chromosomes were not observed, and the metaphase artificial chromosomes appeared to be intact at the level of FISH analysis.

Table 2. Transgenesis rates in murine morulae and blastocysts derived from satellite DNA-based artificial chromosome-injected embryos.

No. of Embryos Analyzed	No. of Embryos Containing the Satellite DNA-Based Artificial Chromosome	Developmental Stage of Positive Embryos	No. of Positive Blastomeres/Total Blastomeres Analyzed
18	8 (44%)	Morula	12/25 (48%)
		Blastocyst	4/8 (50%)
		Blastocyst	2/3 (67%)
		Blastocyst	5/33 (15%)
		Blastocyst	2/26 (8%)
		Blastocyst	6/10 (60%)
		Blastocyst	4/22 (18%)
		Blastocyst	9/23 (39%)

C. Expression of the *lacZ* Gene Contained within the Artificial Chromosomes of Injected Embryos

Transcriptional activity of *lacZ* on the satellite DNA-based artificial chromosomes was investigated in preimplantation mouse embryos injected with artificial chromosomes. Murine zygotes were injected with one artificial chromosome and analyzed for β-galactosidase activity after 4 days in culture. Nine out of 14 embryos (64%), at various stages of development, showed X-gal staining, indicating the presence of a functional marker gene within the artificial chromosome. All embryos exhibited a mosaic expression pattern.

III. Analysis of Founder Mice

Embryos injected with satellite DNA-based artificial chromosomes were implanted into pseudopregnant females. PCR analysis of tail DNA samples revealed that 3 of the 44 mice (7%) born were positive for the *hph* gene (Table

3). One of these was a male that died perinatally (one other PCR-negative pup from the same litter also died perinatally) and the other two (one male, one female) were healthy and phenotypically normal (similar weight/size as non-transgenic siblings or other mice of the same age). Mitogen-activated peripheral blood lymphocytes from the female transgenic founder were analyzed by FISH using an *hph* probe. The chromosome spreads revealed the presence of intact artificial chromosomes in approximately 60% of the cells analyzed (Fig. 1). This pattern was seen in 58% of the metaphase spreads observed, each of which also maintained the normal diploid chromosome number of 40. There was no detectable evidence of translocation of artificial chromosome marker sequences onto host chromosomes.

Table 3. PCR analysis of mice derived from satellite DNA-based artificial chromosome-injected embryos.

Experiment No.	No. of Injected Embryos	No. of 2-Cell Embryos Implanted	No. of Mice Born	No. of *hph* Mice (percentage of positive mice)
1	63	20	8[a]	1[b] male (13%)
2	115	20	9	1 female (11%)
3	50	22	6	0
4	78	17	8	0
5	48	19	7	0
6	39	11	6	1 male (17%)
Total	393	109	44	3 (7%)

[a]Two out of eight pups died perinatally.
[b]Died perinatally.

IV. Analysis of Founder Progeny

The female transgenic founder was mated with wild type (C57BL/6 x CBA) F1 males. Out of six litters, a total of 41 progeny were born, 19 of which were transgenic (46%) as determined by PCR analysis of tail DNA for the presence of the *hph* gene. Of eleven surviving mice, 6 were transgenic (55%); of the 30 mice that died apparently as a result of maternal neglect, 13 were

transgenic (43%), indicating that progeny harboring artificial chromosomes did not have a higher incidence of mortality as compared to progeny without artificial chromosomes. FISH analysis of metaphase spreads of peripheral blood lymphocytes from the artificial chromosome-positive progeny revealed the presence of intact satellite DNA-based artificial chromosomes, with no apparent translocation of artificial chromosome marker DNA onto the host chromosomes.

The artificial chromosome was detected in approximately 60% of the lymphocytes analyzed and this level was maintained stably for many months in both the founder and its progeny (Fig. 1). The finding that not all lymphocytes were artificial chromosome-positive may have been due in part to limitations of the FISH technique wherein the occurrence of false negative cells cannot be accurately assessed. However, we have previously demonstrated that we can detect the satellite DNA-based artificial chromosome in up to 96% of metaphase spreads analyzed in cell lines carrying the same artificial chromosome using the same standard technique [Telenius *et al.* (1999) *Chromosome Res. 7*:3-7].

Mosaicism in founder mice is common in conventional transgenesis methods involving the transfer of naked DNA [Chan *et al.* (1999) *Mol. Reprod. Dev. 52*:406-413]. A delay in the onset of satellite DNA-based artificial chromosome replication after microinjection into the pronucleus may account for the mosaicism observed in peripheral blood tissue. A similar delay in the onset of replication of the artificial chromosome during the first round(s) of embryo cell division might also account for the mosaicism observed in the progeny.

Metaphase spreads obtained from the artificial chromosome-negative siblings of both the founder and its progeny were negative for the presence of the artificial chromosomes by FISH analysis. The growth rates and body size of the positive progeny were comparable to those of non-transgenic littermates. To date, the founder mouse and her six transgenic progeny have not exhibited any overt phenotypic abnormalities.

V. Summary and Conclusions

Using satellite DNA-based artificial chromosomes and methods described in the above-referenced application, it has been possible to generate viable transgenic mice that stably maintain satellite DNA-based artificial chromosomes as discrete elements that remain separate from the host genome. The artificial chromosomes were able to undergo mitotic segregation from early embryonic development in the absence of selective pressure, and did not show evidence of integration into the host genome. The maintenance of the satellite DNA-based artificial chromosomes within cells was sustained into adulthood in the mice, and importantly, these chromosomes were transmitted through the germline, as demonstrated by the presence of these artificial chromosomes in 50% of the founder progeny.

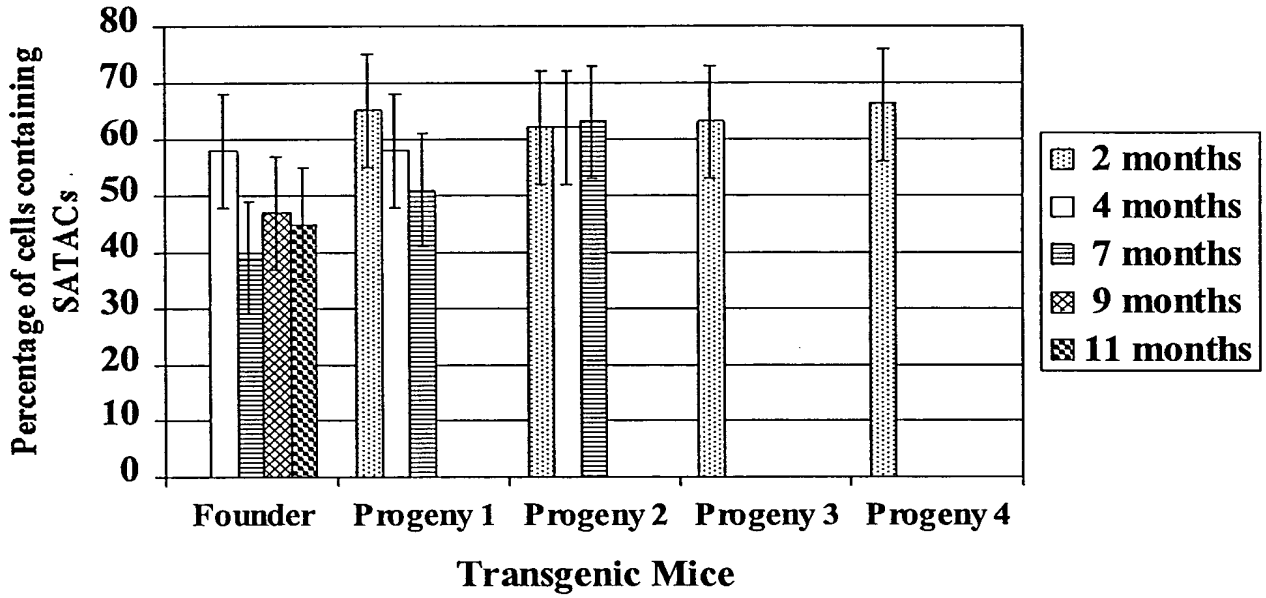
I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Carl F. Perez

Carl Perez

Date: March 6, 2000

Figure 1


Percentage of cells containing SATACs
80
70
60
50
40
30
20
10
0
Founder
Progeny 1
Progeny 2
Progeny 3
Progeny 4
Transgenic Mice
2 months
4 months
7 months
9 months
11 months